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Real-Time Quantitative RT-PCR Analysis of Human Bone Marrow Stromal Cells During Osteogenic Differentiation In Vitro

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Abstract We developed and used real-time RT-PCR assays to investigate how the expression of typical osteoblast-related genes by human bone marrow stromal cells (BMSC) is regulated by (i) the culture time in medium inducing osteogenic differentiation and (ii) the previous expansion in medium enhancing cell osteogenic commitment. BMSC from six healthy donors were expanded in medium without (CTR) or with fibroblast growth factor-2 and dexamethasone (FGF/Dex; these factors are known to increase BMSC osteogenic commitment) and further cultivated for up to 20 days with ascorbic acid, β-glycerophosphate and dexamethasone (these factors are typically used to induce BMSC osteogenic differentiation). Despite a high variability in the gene expression levels among different individuals, we identified the following statistically significant patterns. The mRNA levels of bone morphogenetic protein-2 (BMP-2), bone sialo protein-II (BSP), osteopontin (OP) and to a lower extent cbfa-1 increased with culture time in osteogenic medium (OM), both in CTR- and FGF/Dex-expanded BMSC, unlike levels of alkaline phosphatase, collagen type I, osteocalcin, and osteonectin. After 20 days culture in OM, BMP-2, BSP, and OP were more expressed in FGF/Dex than in CTR-expanded BMSC (mRNA levels were, respectively, 9.5-, 14.9-, and 5.8-fold higher), unlike all the other investigated genes. Analysis of single-colony-derived strains of BMSC further revealed that after 20 days culture in OM, only a subset of FGF/Dex-expanded clones expressed higher mRNA levels of BMP-2, BSP, and OP than CTR-expanded clones. In conclusion, we provide evidence that mRNA levels of BMP-2, BSP, and OP, quantified using real-time RT-PCR, can be used as markers to monitor the extent of BMSC osteogenic differentiation in vitro; using those markers, we further demonstrated that only a few subpopulations of BMSC display enhanced osteogenic differentiation following FGF/Dex expansion. J. Cell. Biochem. 85:737–746, 2002.

Key words: mesenchymal stem cells; clones; bone morphogenetic protein; bone sialoprotein; osteopontin

Bone marrow stromal cells (BMSC) represent a phenotypically and functionally heterogeneous population of mesenchymal precursors providing support for haematopoiesis [Dexter et al., 1977] and contributing to the physiological regeneration of bone, cartilage, adipose, muscle, and other connective tissues [Owen, 1988; Beresford, 1989]. Ex vivo expanded BMSC have been used in several in vivo model systems to generate bone tissue, and when associated with carriers enabling host vascularization, were found to form 'ossicles' resembling the architecture of the normal bone/bone marrow organ [Friedenstein and Kuralesova, 1971; Goshima et al., 1991; Martin et al., 1997]. BMSC can also be directed towards the osteogenic lineage in vitro, if cultured in the presence of dexamethasone, β-glycerophosphate and ascorbic acid [Maniatopoulos et al., 1988]. BMSC osteogenic differentiation in vitro is generally characterized by appearance of osteoblastic cell morphology, increased alkaline phosphatase (AP) activity, and formation of

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mineralized bone-like nodules containing collagen type I (Coll I), osteocalcin (OC), osteonectin (ON), bone sialo protein-II (BSP), and osteopontin (OP) [Maniatopoulos et al., 1988; Jaiswal et al., 1997; Malaval et al., 1999].

The mRNA expression of the genes encoding the above mentioned proteins during the osteogenic differentiation of human BMSC in vitro has not yet been systematically investigated, and results from different studies are sometimes contradictory. As an example, one group found that mRNA levels for OC and OP increased with time in culture [Byers et al., 1999], whereas other studies reported a decrease in the mRNA expression of OP [Malaval et al., 1994; Rickard et al., 1996] and OC [Cheng et al., 1996] during BMSC differentiation. Identification of mRNA markers characterizing the progression of human BMSC towards the osteogenic lineage is further complicated by the known variability of cells from different individuals, harvested using different methods [Phinney et al., 1999], and by limitations inherent to the techniques used (i.e., semi-quantitative techniques like Northern blots or conventional RT-PCR).

The recently established real-time quantitative RT-PCR technology has made mRNA analysis more reproducible, precise, and sensitive than conventional RT-PCR, because it allows measuring the amount of amplified product (i) using a quantitative laser-based method and (ii) in the early exponential phase of the PCR reaction, when none of the reagents is rate-limiting [Gibson et al., 1996]. In this study, we developed real-time quantitative RT-PCR assays for genes encoding the most typical osteoblast-related membrane and extracellular matrix molecules (i.e., AP, Coll I, OC, ON, OP, BSP), as well as for one of the most potent growth factors involved in recruitment and differentiation of mesenchymal precursors (i.e., bone morphogenetic protein-2 (BMP-2)) and for the main transcription factor related to osteogenesis (i.e., core binding factor A1 (cbfa-1)). We then used the developed assays to quantify how the expression of these genes is regulated in human BMSC by (i) the culture time in medium inducing osteogenic differentiation and (ii) the cell expansion in medium supplemented with FGF-2 and dexamethasone (FGF/Dex), previously shown to enhance BMSC osteogenic commitment [Muraglia et al., 1998; Walsh et al., 2000]. In order to identify relevant patterns, human BMSC were harvested under standardized conditions from multiple healthy donors in the same age range. Furthermore, since the differentiation ability of BMSC is known to be heterogeneous across different subpopulations within the same primary culture [Malaval et al., 1994; Satomura et al., 2000], we used the method on both single- and multi-colony-derived strains of BMSC.

MATERIALS AND METHODS

Methods

Human material. Bone marrow aspirates and trabecular bone chips were obtained during routine orthopedic surgical procedures involving exposure of the iliac crest, after informed consent. Marrow aspirates (20 ml volumes) were harvested from six healthy donors (female, 32–51 years) using a bone marrow biopsy needle inserted through the cortical bone; aspirates were immediately transferred into plastic tubes containing 15,000 IU heparin. Trabecular bone chips (10–30 mm^3 particles) were obtained from five healthy donors (female, 42–48 years).

Isolation and expansion of BMSC. After diluting the marrow aspirates with phosphate-buffered saline (PBS) at a ratio of 1:4, nucleated cells were isolated using a density gradient solution (Histopaque®, Sigma Chemical, Buchs, CH). Control (CTR) medium consisted of Dulbecco's modified Eagle medium (DMEM) with 10% fetal bovine serum (lot 40G2470J, GIBCO-BRL Life Technologies, Basel, CH), 4.5 mg/ml D-Glucose, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 100 mM HEPES buffer, 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.29 mg/ml L-glutamine. Nucleated cells were plated at a density of 100,000 cells/cm^2 in either CTR medium or CTR medium supplemented with 5 ng/ml fibroblast growth factor-2 (R&D Systems, Wiesbaden, D) and 10 nM dexamethasone (Sigma) (FGF/Dex) and cultured in a humidified 37°C/5% CO_2 incubator. Medium was changed twice in a week. BMSC were selected on the basis of adhesion and proliferation on the plastic substrate.

Osteogenic stimulation. Upon reaching subconfluency, BMSC were detached using 0.05% trypsin/0.53 mM EDTA (GIBCO-BRL, CH) and plated in 12-well tissue culture dishes at a density of 3,000 cells/cm^2. Cells were cultured in osteogenic medium (OM), consisting of CTR medium supplemented with 10 nM Dex,

0.1 mM L-ascorbic acid-2-phosphate and 10 mM β-glycerophosphate [Maniatopoulos et al., 1988]. Cultures were harvested at timed intervals (3, 7, 11, 15, and 20 days) and processed for biochemical, histological, and mRNA analysis as described below.

Culture of BMSC clones. Single-colony-derived strains (clones) of BMSC were obtained from one donor (34 years old) by limiting dilution [Muraglia et al., 2000]. Briefly, nucleated cells from the bone marrow aspirate were seeded in 96-well plates at a density of 2,000 cells/well in CTR or FGF/Dex supplemented medium. Cell layers from wells containing a colony were passaged in 24-well plates and split into two fractions when subconfluent. One fraction was further expanded in 5 cm^2 dishes and stained for AP as described below, whereas the second fraction was cultured for 20 days in OM and processed for mRNA analysis.

Isolation of osteoblasts. Bone chips were flushed extensively with PBS and incubated with 2 mg/ml collagenase A (Boehringer Mannheim, Mannheim, D) for 30 min at 37°C on a 3D orbital shaker (Bioblock Scientific, Frenkendorf, CH) [Byers et al., 1999]. The medium was discarded and the digestion step was repeated under identical conditions using fresh collagenase. Released cells were then plated in OM and processed for RNA analysis when subconfluent. In this article, osteoblast-like cells isolated according to this method will be referred to as 'osteoblasts', although a direct comparison with the functional state of osteoblasts in vivo has not been made.

Analytical Methods

AP and DNA assays. Cultures were rinsed twice with PBS, and the cell layer was scraped in 0.01% sodium dodecyl sulfate. AP activity was measured in duplicate aliquots as the rate of conversion of *p*-nitrophenyl phosphate using Sigma kit 104. DNA amounts were measured in duplicate aliquots of the same samples using the CyQuant cell proliferation assay kit (Molecular Probes, Leiden, NL), according to the manufacturer's instructions. AP activity, expressed as nanomoles of *p*-nitrophenol/h/well, was normalized to the DNA amount.

Calcium assay. Total calcium was measured using Sigma Kit 587 after rinsing and extracting the cell layer in 0.5 N HCl as described elsewhere [Jaiswal et al., 1997]. The amount of deposited calcium was expressed as μg/dish.

Proliferation rate. The number of doublings of single-colony-derived strains of BMSC during the expansion phase was determined as the logarithm in base 2 of the number of cells counted after expansion. The proliferation rate of BMSC was defined as the number of doublings during the expansion phase divided by the time required for the expansion, and was expressed as doublings/day.

Histochemical analyses. Single-colony-derived strains of BMSC were stained for AP using Sigma Kit 86-C and macroscopically evaluated. Calcium deposits were stained using silver nitrate as follows. Cell layers were fixed in 10% formalin, incubated in 5% aqueous silver nitrate, and exposed to UV light at 365 nm (UV Stratalinker™ 1800, Stratagene GmbH, Zürich, CH) for 30 min. Samples were then washed with deionized water and excess silver was removed by rinsing with 5% aqueous sodium thiosulfate.

Total RNA extraction and cDNA synthesis. RNA was extracted from cell layers using Trizol (Life Technologies) according to the single step acid-phenol guanidinium method [Chomczynski and Sacchi, 1987]. RNA was treated with DNaseI using the DNA-free™ kit (AMS Biotechnology Ltd., CH) and quantified spectrometrically. Starting from 2–3 μg RNA, 20 μl cDNA were synthesized using 500 μg/ml random hexamers (Catalys AG, CH) and 1 μl of 50 U/ml Stratascript™ reverse transcriptase (Stratagene, NL), in the presence of dNTPs. cDNA was then diluted to 200 μl using DNAse-free water.

Real-time quantitative RT-PCR. Reactions were performed and monitored using a ABI Prism 7700 Sequence Detection System (Applied Biosystems, Rotkreuz, Switzerland, CH). The PCR 2 × master mix was based on AmpliTaq Gold DNA polymerase (Applied Biosystems). In the same reaction, cDNA samples (5 μl for a total volume of 25 μl per reaction) were analyzed both for the gene of interest and the reference gene (18S rRNA), using a multiplex approach (Perkin Elmer User Bulletin N. 2). The probe for 18S rRNA was fluorescently labeled with VIC™ and TAMRA (Applied Biosystems), whereas, probes for the genes of interest were labeled with 6-carboxy-fluorescein (FAM) and TAMRA. Cycle temperatures and times were as previously described [Martin et al., 2001]. The 18S rRNA was selected as a reference gene based on preliminary

experiments, indicating its higher stability of expression by BMSC as compared to GAPDH. Expression levels for each gene of interest were calculated by normalizing the quantified mRNA amount to the 18S rRNA and by further dividing the resulting value by that obtained in human osteoblasts (average of five donors) using an identical procedure ($2^{\Delta\Delta Ct}$ formula, Perkin Elmer User Bulletin No. 2). Each sample was assessed at least in duplicate.

Primers and probes. Primers and probes for human AP, BMP-2, BSP, cbfa1, Coll I, OC, ON, and OP were designed with the assistance of Primer Express™ software Version 1.0 (Applied Biosystems). For each gene of interest, one primer or the probe was placed at the junction between two exons. Primers and probe for the 18S sequence were designed by Applied Biosystems. The efficiency of each set of primers and probes, assessed as previously described [Martin et al., 2001], was always higher than 90%. Primers were purchased from Microsynth (Balgach, CH) and probes were from Applied Biosystems or Eurogentech (Seraing, B). The nucleotide sequences of the oligonucleotide hybridization primers and probes are shown in Table I.

Statistical Analyses

All values are presented as mean ± standard error. Statistics were assessed using a Student's *t*-test, assuming double-sided independent variance and with $P < 0.05$ considered to be significant. Correlation analysis was performed using Pearson's test (SPSS 8.0, Chicago, IL).

RESULTS

Biochemical Analyses

During 20 days of culture in OM, CTR-, and FGF/Dex-expanded BMSC underwent a total of 3–4 doublings and proliferated at a comparable rate, as assessed by the measured DNA at defined time points (Fig. 1A).

AP activity, normalized to the DNA amount, reached maximal levels in the first week of culture in OM. In this early phase of differentiation, FGF/Dex-expanded cells displayed a sharper increase in AP activity and reached higher levels than CTR-expanded cells. After the first week in OM, AP activities of FGF/Dex-expanded cells decreased, reaching levels comparable to those of CTR-expanded BMSC (Fig. 1B).

The accumulation of calcium in the extracellular matrix was negligible during the first few days in OM and then steadily increased with time, at a comparable rate in CTR- and FGF/Dex-expanded BMSC (Fig. 1C). The progressive increase of mineralization with time was confirmed by silver nitrate stain of parallel cultures, indicating the formation of bone-like nodules (data not shown).

TABLE I. Description of the Designed Primes and Probes

Gene	Forward and reverse primers (5′→3′)	Primer concentration used (nM)	Probe[a] (5′→3′)	Probe concentration used (nM)
18S rRNA	CGGCTACCACATCCAAGGAA GCTGGAATTACCGCGGCT	25	TGCTGGCACCAG ACTTGCCCTC	50
AP	GACCCTTGACCCCCACAAT GCTCGTACTGCATGTCCCCT	300	TGGACTACCTATTGGGTC TCTTCGAGCCA	100
BSP	TGCCTTGAGCCTGCTTCC GCAAAATTAAAGCAGTCTT- CATTTTG	300	CTCCAGGACTGCCAGAG GAAGCAATCA	100
BMP-2	AACACTGTGCGCAGCTTCC CTCCGGGTTGTTTTCCCAC	300	CCATGAAGAATCTTTGGA AGAACTACCAGAAACTG	100
cbfa1	GCCTTCAAGGTGGTAGCCC CGTTACCCGCCATGACAGTA	300	CCACAGTCCCATCTGGTA CCTCTCCG	100
Coll I	CAGCCGCTTCACCTACAGC TTTTGTATTCAAT- CACTGTCTTGCC	300	CCGGTGTGACTCGTGCAG CCATC	100
OC	GAAGCCCAGCGGTGCA CACTACCTCGCTGCCCTCC	300	TGGACACAAAGGCTGCA CCTTTGCT	150
ON	ATCTTCCCTGTACACTGG- CAGTTC CTCGGTGTGGGAGAGG- TACC	300	CAGCTGGACCAGCACCC CATTGAC	100
OP	CTCAGGCCAGTTGCAGCC CAAAAGCAAATCACTG- CAATTCTC	500	AAACGCCGACCAAGGAA AACTCACTACC	200

[a]The underlined bases in the primer or probe sequence indicate the position of an intron in the corresponding genomic sequence.

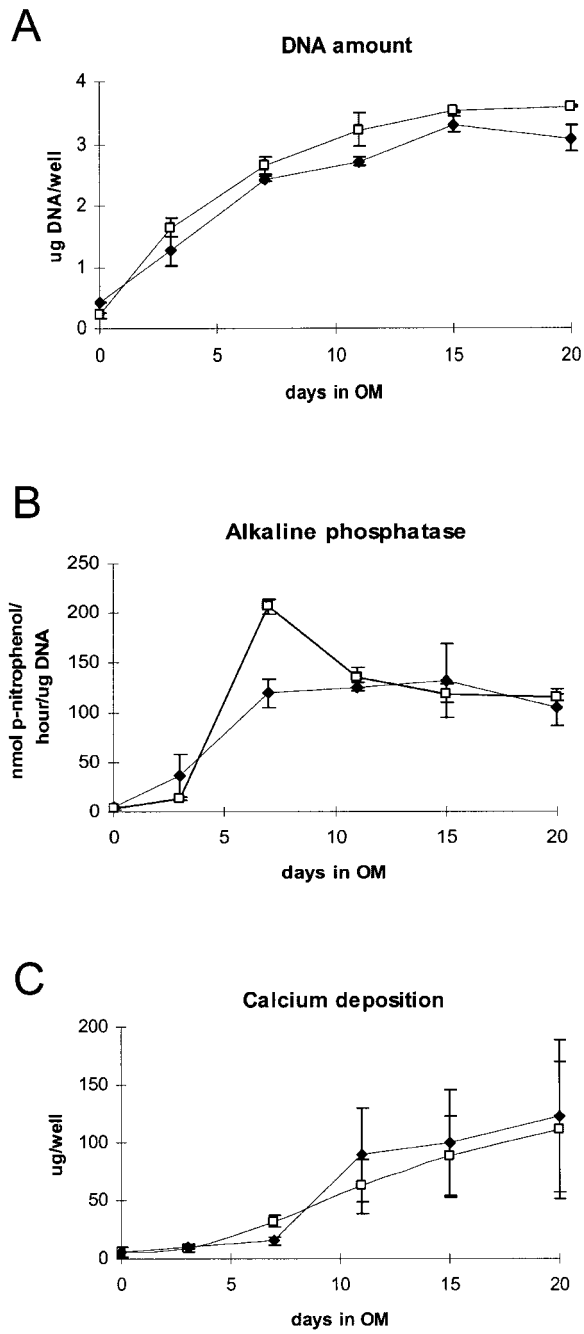

Fig. 1. DNA amount (**A**), AP activity (**B**), and calcium deposition (**C**) measured during culture of BMSC in OM. Cells from two donors were previously expanded in medium without (CTR, ◆) or with the combination of FGF-2 and dexamethasone (FGF/Dex, □).

Real-Time RT-PCR Assays

CTR- and FGF/Dex-expanded BMSC from two donors (40 and 41 years of age) were assessed for the expression of all the genes of interest at different times during culture in OM (Fig. 2). The mRNA levels of CTR-expanded BMSC from the two donors differed substantially from each other (Fig. 2, black symbols), both at the early culture phases (with the exception of values for Coll I) and during cultivation time in OM. FGF/Dex expansion induced changes in the mRNA expression levels that were markedly different in BMSC cultures from the two donors (Fig. 2, empty symbols). As an example, FGF/Dex-expanded BMSC from 'donor 1' expressed levels of AP that were lower than in CTR-expanded BMSC in the late culture phase, whereas an opposite trend was observed for cells from 'donor 2' (Fig. 2A). Only BMP-2 expression levels were consistently higher in FGF/Dex- than in CTR-expanded BMSC (Fig. 2B).

Due to the high variability among the different donors, in the following experiments we used BMSC cultures from six individuals and introduced different normalizations of the mRNA expression levels, according to the specific question addressed.

In order to determine if the expression of the investigated genes was regulated by the culture time in OM, mRNA levels quantified after 20 days culture in OM were divided by those measured after 3 days culture in OM. Of all the genes of interest, only BMP-2, BSP, OP, and to a lower extent, cbfa-1 were significantly more expressed after 20 days than after 3 days culture in OM, both for CTR- and FGF/Dex-expanded BMSC (Fig. 3A). For CTR-expanded cells, the increase averaged 13.7 ± 4.0-, 8.2 ± 1.9-, 13.7 ± 6.5-, and 2.6 ± 0.4-fold respectively, for BMP-2, BSP, OP, and cbfa-1. None of the genes of interest was significantly downregulated during culture in OM.

In order to determine if the expression of the investigated genes was regulated by the previous cell expansion in medium supplemented with FGF/Dex, mRNA levels quantified after 20 days culture in OM in FGF/Dex-expanded cells were divided by those measured in parallel cultures of CTR-expanded cells. Only BMP-2, BSP, and OP mRNA levels were significantly higher in FGF/Dex-expanded cells, averaging, respectively, 9.5 ± 1.7-, 14.9 ± 4.8-, and 5.8 ± 1.8-fold the levels of CTR-expanded cells (Fig. 3B). None of the genes of interest was significantly downregulated in FGF/Dex- as compared to CTR-expanded BMSC.

Analysis of BMSC Clones

We then decided to determine if the mRNA expression of BMP-2, BSP, and OP after culture

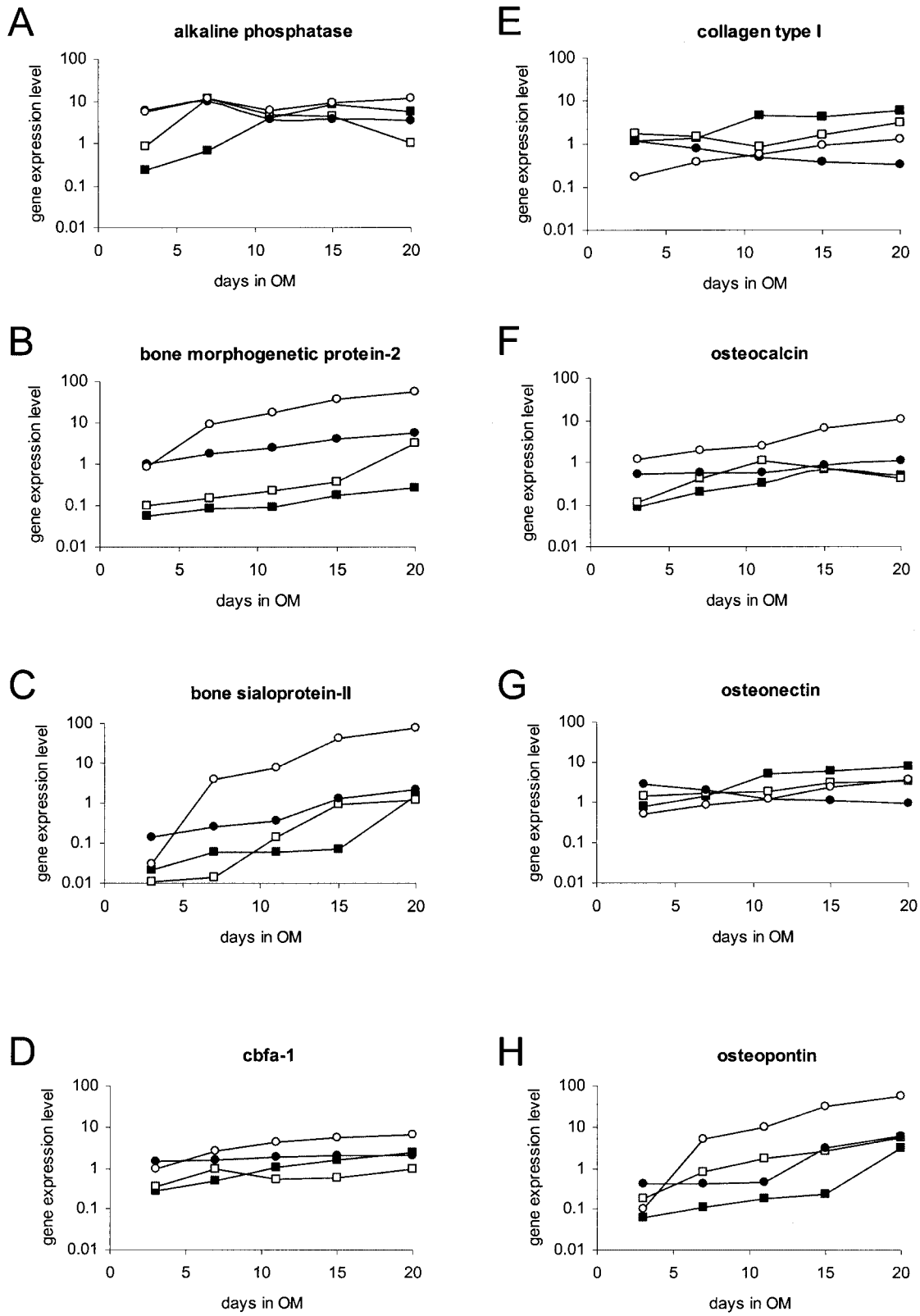

Fig. 2. Representative curves of the mRNA levels of AP (**A**), BMP-2 (**B**), BSP (**C**), cbfa-1 (**D**), Coll I (**E**), OC (**F**), ON (**G**), and OP (**H**) quantified during BMSC culture in OM. Cells from two different donors (squares, donor 1; circles, donor 2) were previously expanded without (CTR, black symbols) or with the combination of FGF-2 and dexamethasone (FGF/Dex, empty symbols). Expression levels are expressed as fold of those measured in human primary osteoblasts (average of five different donors).

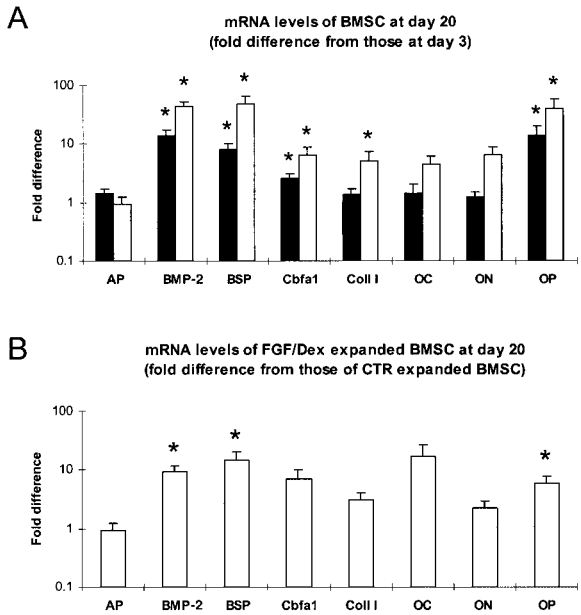

Fig. 3. Levels of AP, BMP-2, BSP, cbfa-1, Coll I, OC, ON, and OP mRNA measured in BMSC from six independent donors, expanded without (CTR, black bars) or with FGF-2 and dexamethasone (FGF/Dex, empty bars). In (**A**), mRNA levels measured in cells cultured for 20 days in OM are expressed as fold difference with respect to levels measured in the same cells cultured for 3 days in the same medium. In (**B**), mRNA levels measured in FGF/Dex-expanded cells cultured for 20 days in OM are expressed as fold difference with respect to levels measured in CTR-expanded cells cultured for the same time in the same medium. Asterisks indicate statistically significant differences.

in OM is upregulated by previous FGF/Dex expansion in a similar fashion across different BMSC subpopulations. Using BMSC from one donor, we isolated and expanded 26 clones in CTR medium and 23 clones in FGF/Dex supplemented medium. The proliferation rate of clones significantly increased from 0.54 ± 0.02 doublings/day in CTR medium to 0.69 ± 0.03 doublings/day in FGF/Dex supplemented medium ($P < 0.001$). The fraction of AP stained clones at the end of the expansion phase was similar in CTR- and FGF/Dex-expanded clones (75 and 80%, respectively). A total of 15 clones expanded in CTR medium and 15 clones expanded with FGF/Dex was further cultured in OM for 20 days and assessed by real-time RT-PCR. We observed a very high variability (up to 5–6 orders of magnitude) in the mRNA expression of BMP-2, BSP, and OP among various clones, even if expanded under the same condition (Table II). Due to this high variability, we found no statistical difference in the average mRNA levels of BMP-2, BSP, or OP expressed by CTR- and FGF/Dex-expanded clones. However, while the lowest expression levels of BMP-2, BSP, and OP were comparable in clones expanded with or without FGF/Dex, the highest expression levels were markedly higher in clones expanded in the presence of FGF/Dex (Table II). Figure 4 displays the mRNA levels of BMP-2 quantified in all 30 clones; the diagram indicates that both CTR- and FGF/Dex-expanded clones covered the corresponding range of expression levels, and confirms that such range started from comparable levels in CTR- and FGF/Dex-expanded clones, but extended to higher levels in FGF/Dex-expanded clones. The percentages of FGF/Dex-expanded clones expressing mRNA levels of BMP-2, BSP, and OP higher than the highest levels expressed by CTR-expanded clones were 33, 20, and 18%, respectively. The average BMP-2, BSP, and OP mRNA levels reached by these groups of FGF/Dex-expanded clones were, respectively, 30.6-, 12.3-, and 2.5-fold higher than the highest levels reached by CTR-expanded clones. There was a significant correlation between levels of mRNA expression of BMP-2, BSP, and OP in the same clones, either if CTR- or FGF/Dex-expanded (Table III), as assessed by Pearson's correlation tests. Finally, we found that neither the proliferation rates of clones during the expansion phase nor their AP activity after expansion were correlated with the levels of expression of BMP-2, BSP, or OP after culture in OM.

TABLE II. Lowest and Highest mRNA Expression Levels in BMSC Clones

Gene	Expansion condition	Expression levels[a]	
		Lowest	Highest
BMP-2	CTR	0.0088	8.77
	FGF/Dex	0.0070	622.75
BSP	CTR	0.0002	78.93
	FGF/Dex	0.0005	1707.30
OP	CTR	0.0132	274.85
	FGF/Dex	0.0043	990.83

[a]Expression levels are expressed as fold of those measured in primary human osteoblasts (average of five donors).

DISCUSSION

In the present study, we describe for the first time the development and use of real-time RT-PCR assays to quantify the expression of osteoblast-related genes in human BMSC. Interestingly, of all the genes investigated, only BMP-2, BSP, and OP were significantly

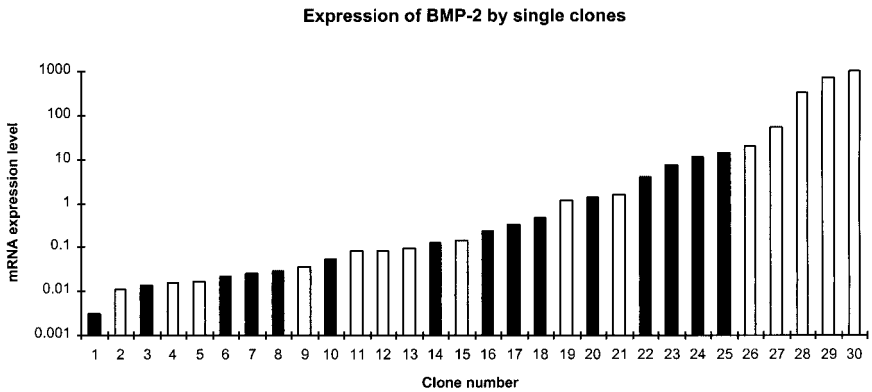

Fig. 4. Levels of BMP-2 mRNA expressed by 30 different clonal populations of BMSC after culture in OM for 20 days. Clones were expanded without (CTR, black bars) or with FGF-2 and dexamethasone (FGF/Dex, empty bars). Expression levels are expressed as fold of those measured in human primary osteoblasts (average of five donors).

upregulated both by culture time in OM and by cell expansion in medium containing FGF/Dex. We further demonstrated that after culture in OM, only a few clonal subpopulations of BMSC display enhanced expression of these genes, if previously expanded in the presence of FGF/Dex.

Our in vitro model system comprises two culture phases: BMSC are first expanded with or without FGF/Dex and then further cultured in an identical medium, inducing differentiation towards the osteoblast lineage. This culture method is different from most others described in literature, where cells are directly exposed to OM after isolation from the bone marrow, and parallels that of a previous study, where BMSC were first expanded with or without FGF/Dex and then implanted in nude mice to study their ability to generate ectopical bone in vivo [Muraglia et al., 1998]. Together with the previous demonstration that BMSC expanded with FGF/Dex have a higher osteogenic commitment and bone forming capacity [Muraglia et al., 1998], our findings suggest that the elevated mRNA expression of BMP-2, BSP, and OP by BMSC in vitro is related to a higher osteogenic ability of the cells. We also found that neither AP activity and mineralization, nor the mRNA expression of AP, cbfa1, Coll I, OC, and ON was significantly different in CTR- and FGF/Dex-expanded BMSC after culture in OM. These results are consistent with previous studies reporting that (i) elevated mRNA expression of BSP in vitro is associated with the capacity for bone formation by murine BMSC [Satomura et al., 2000]; (ii) expression of cbfa1, although essential for osteogenesis during development and postnatal bone formation [Ducy et al., 1997; Komori et al., 1997], is a basic property of functionally different BMSC [Satomura et al., 2000]; (iii) expression of OC by clonal populations of BMSC does not reflect their in vivo bone forming ability [Kuznetsov et al., 1997]; and (iv) the formation of bone-like nodules in vitro is not indicative of the actual cell osteogenic capacity in vivo [Satomura et al., 2000].

TABLE III. Correlation Between the Expression Levels of BMP-2, BSP, and OP in Different Clones[a]

Expansion condition	BMP-2/BSP	BMP-2/OP	BSP/OP
CTR	0.994*	0.869*	0.921*
FGF/Dex	0.998*	0.926*	0.942*

[a]The mRNA levels of BMP-2, BSP, and OP expressed by the same clones (either CTR- or FGF/Dex-expanded) were correlated using Pearson's two-tailed test.
*$P < 0.01$.

BMP-2 is a molecule capable of attracting, proliferating, and differentiating mesenchymal progenitor cells, resulting in the transient formation of cartilage and production of bone tissue even at ectopic sites [Wozney, 1989]. BMP-2 has been reported to be expressed by BMSC in vitro, particularly when stimulated with OM [Bi et al., 1999], and in turn to stimulate BMSC to generate bone tissue in vivo [Yamagiwa et al., 2001]. BSP has been proposed as the main nucleator of hydroxyapatite crystal formation and correlates with the initial phase of matrix mineralization [Bianco et al., 1993]. Its expression, which is induced in newly-formed osteoblasts, is

upregulated by hormones and cytokines that promote bone formation and downregulated by factors that suppress bone formation [Ganss et al., 1999]. OP is one of the most abundant noncollagenous proteins in bone; it binds to various extracellular molecules, including type I collagen, fibronectin, OC, and may add physical strength to extracellular matrices [Denhardt and Noda, 1998]. OP has been shown to play an important role in bone remodeling [Denhardt and Noda, 1998], and reduced OP mRNA levels in BMSC were correlated with the reduced bone formation in osteopenia and osteoporosis [Zhang et al., 1995]. Our finding that BMP-2, BSP, and OP are expressed at higher levels by BMSC expanded with FGF/Dex may explain the increased bone-forming capacity of FGF/Dex-expanded cells in vivo [Muraglia et al., 1998]. In fact, a higher expression of BMP-2, BSP, and OP by BMSC would facilitate some of the early events required for bone matrix deposition in vivo, e.g., biosynthetic activity of osteoprogenitor cells, nucleation of hydroxyapatite crystals and functional organization of extracellular matrix molecules. Since it was previously shown that BMP-2 increases the mRNA levels and protein synthesis of BSP and OP [Lecanda et al., 1997], it is possible that elevated expression of BSP and OP in FGF/Dex-expanded BMSC are a consequence of the autocrine effect of BMP-2.

We further investigated how the expression of specific osteogenic markers in the multiclonal population is reflected in single-colony-derived strains of BMSC. We expanded a total of 49 clones with or without FGF/Dex and assessed their proliferation and AP activity during expansion and the profiles of gene expression after culture in OM. In all parameters assessed, we observed a striking heterogeneity among clones, both CTR- and FGF/Dex-expanded. This result is in line with recent studies, indicating a heterogeneous capacity of proliferation [Satomura et al., 2000], differentiation [Aubin, 1998; Muraglia et al., 1998], and osteogenesis [Kuznetsov et al., 1997; Satomura et al., 2000] across different clonal populations of BMSC. We found no significant difference in the average expression of BMP-2, BSP, and OP in CTR- and FGF/Dex-expanded clones after culture in OM, due to the fact that the majority of FGF/Dex-expanded clones behaved similarly to CTR-expanded clones. However, a fraction of the FGF/Dex-expanded clones, ranging between 18 and 33%, expressed mRNA levels of BMP-2, BSP, and OP markedly higher than the highest measured in CTR-expanded clones, possibly accounting for the significant difference in the expression of the three genes measured at the multiclonal population level. The high correlation found in the expression levels of BMP-2, BSP, and OP among clones indicates that the same clones expressed high levels of all the three different genes. Taken together, these data suggest that only a subset of BMSC clones displayed enhanced osteogenic differentiation following expansion with FGF/Dex. It would be interesting to identify markers and/or properties of clones responsive to FGF/Dex during expansion, possibly in order to select a population of more osteogenically-committed cells. In this regard, our results showed that neither AP activity nor proliferation rate can be used as such markers, consistent with a previous study [Kuznetsov et al., 1997].

In conclusion, we have used real-time RT-PCR to quantitatively characterize the osteogenic differentiation of human BMSC in vitro, both at multiclonal and clonal levels. Our main finding is that the mRNA expression of the genes encoding BMP-2, BSP, and OP quantitatively characterizes the progression and extent of BMSC osteogenic differentiation in vitro. The developed assays and the defined markers of differentiation can be used to investigate how the progression of mesenchymal progenitor cells into the osteogenic lineage is modulated by a variety of other factors of different nature (e.g., interaction with different biomaterials, exposure to bioactive molecules, or application of physical forces).

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